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RESEARCH ARTICLE

Genomic variation across the Yellow-rumped Warbler species complex

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ABSTRACT

Populations that have experienced long periods of geographic isolation will diverge over time. The application of high-throughput sequencing technologies to study the genomes of related taxa now allows us to quantify, at a fine scale, the consequences of this divergence across the genome. Throughout a number of studies, a notable pattern has emerged. In many cases, estimates of differentiation across the genome are strongly heterogeneous; however, the evolutionary processes driving this striking pattern are still unclear. Here we quantified genomic variation across several groups within the Yellow-rumped Warbler species complex (*Setophaga* spp.), a group of North and Central American wood warblers. We showed that genomic variation is highly heterogeneous between some taxa and that these regions of high differentiation are relatively small compared to those in other study systems. We found that the clusters of highly differentiated markers between taxa occur in gene-rich regions of the genome and exhibit low within-population diversity. We suggest these patterns are consistent with selection, shaping genomic divergence in similar genomic regions across the different populations. Our study also confirms previous results relying on fewer genetic markers that several of the phenotypically distinct groups in the system are also genomically highly differentiated, likely to the point of full species status.

Keywords: evolutionary genomics, hybridization, gene flow, genotyping-by-sequencing, speciation, natural selection

Variación genómica a través del complejo de especies de *Setophaga coronata*

RESUMEN

Las poblaciones que han experimentado largos periodos de aislamiento geográfico se diferenciarán con el paso del tiempo. La aplicación de tecnologías de secuenciación de alto rendimiento para el estudio de los genomas de taxones relacionados ahora nos permite cuantificar a escala fina las consecuencias de esta divergencia s través del genoma. Luego de numerosos estudios emerge un patrón notable: en muchos casos los estimados de diferenciación a través del genoma son fuertemente heterogéneos. Sin embargo, los procesos evolutivos que gobiernan este patrón aún no son claros. En este estudio cuantificamos la variación genómica a través de varios grupos dentro del complejo de especies de *Setophaga coronata*, un grupo de reinitas de Norte y Centroamérica. Mostramos que la variación genómica es altamente heterogénea entre algunos de los taxones y que las regiones de alta diferenciación son relativamente pequeñas en comparación con otros sistemas de estudio. Encontramos que las agrupaciones de marcadores áltamente diferenciados entre taxones se encuentran en regiones del genoma ricas en genes y también muestran baja diversidad intrapoblacional. Sugerimos que estos patrones son consistentes con un efecto de procesos de selección natural sobre la divergencia genómica en regiones genómicas similares a través de las diferentes poblaciones. Nuestro estudio también confirma resultados previos basados en pocos marcadores genéticos en los que se determinó que muchos de los grupos fenotípicamente distintos en este sistema también están áltamente diferenciados en sus genomas, probablemente al punto en que pueden ser consideradas con el estatus de especie.

Palabras clave: especiación, flujo genético, genómica evolutiva, genotipado por secuenciación, hibridación, selección natural

INTRODUCTION

Recent studies of closely related avian species pairs have revealed that genetic divergence varies greatly between different portions of the genome (Parchman et al. 2013, Poelstra et al. 2014, Seehausen et al. 2014, Burri et al. 2015, Delmore et al. 2015, reviewed in Toews et al. 2016a). The causes of this genome-wide heterogeneity are controversial (Cruickshank and Hanh 2014, Burri et al. 2015, Delmore et al. 2015, Payseur and Rieseberg 2016, Toews et al. 2016a). A common interpretation across these genome scans between avian species is that elevated divergence between populations is the result of genomically localized natural selection (Burri et al. 2015, Delmore et al. 2015). While there is still debate as to the nature of selection (i.e. whether it is positive or purifying), this controversy stems primarily from the difficulty in interpreting the signature of selection from molecular data alone (Payseur and Rieseberg 2016).

Another notable pattern that has emerged from genome scans across different avian species groups relates to the size and extent of regions of divergence. For example, in some comparisons, such as between the European Pied Flycatcher (*Ficedula hypoleuca*) and Collared Flycatcher (*F. albicollis*; Ellegren et al. 2012, Burri et al. 2015) and between Inland Swainson's Thrush (*Catharus ustulatus swainsoni*) and Coastal Swainson's Thrush (*Catharus ustulatus ustulatus*; Ruegg et al. 2014, Delmore et al. 2015), regions of divergence are large. In these systems, peaks of divergence can, in some cases, comprise more than half of a chromosome (e.g., some divergence peaks are >25 Mb wide). By contrast, regions of divergence in other avian pairs are fewer in number and much smaller. This is the case between the Hooded Crow (*Corvus cornix*) and Carrion Crow (*Corvus corone*; Poelstra et al. 2014) and between the Golden-winged Warbler (*Vermivora chrysoptera*) and Blue-winged Warbler (*Vermivora cyanoptera*; Toews et al. 2016b), where the few regions of elevated divergence are only tens or hundreds of thousands of base pairs wide.

What are the important differences among these pairs that contribute to the variation in the number and size of these divergence regions? The answer likely relates to variation in the time of splitting from a common ancestor, the history of selection, hybridization, and the role that the recombination landscape plays in shaping patterns of divergence in different systems (Burri et al. 2015). Hampering general characterizations across avian taxa is the limited number of systems where these kinds of genomic data have been assayed, although these are increasing with the continued application of genomic approaches to non-model avian groups (Toews et al. 2016a).

Here we add to this growing literature by conducting a genome-wide assay of variation in another important avian group, the Yellow-rumped Warbler species complex (*Setophaga* spp.). Our study focused on quantifying genomic patterns of divergence between the closely related taxa in this system. In particular, we were interested in understanding how large the regions of divergence may be between these groups, allowing us to compare and contrast these patterns with similar studies of other avian taxa. Using a variety of other bioinformatic tools and analyses, we also provide a preliminary exploration of the evolutionary processes that might have contributed to the genomic patterns. A subsequent goal with this new genomic data is to elucidate the evolutionary history of this group and thus provide additional resolution to the taxonomic boundaries among taxa, which has challenged nomenclature committees. For example, the complex is currently treated as a single species, *Setophaga coronata*, by the American Ornithologists' Union, but as 3 species by the International Ornithological Committee (IOC); we used the IOC taxonomy in this article.

The Yellow-rumped Warbler system illustrates a wide spectrum of genetic and phenotypic divergence and evidence of extensive hybridization in certain areas of secondary contact (Hubbard 1969, Barrowclough 1980, Milá et al. 2008, Brelsford and Irwin 2009, Brelsford et al. 2011, Toews et al. 2014b). Previous genetic work in the system suggests that this system consists of a complex set of several geographically distinct groups, with hybridization between some of them (Brelsford et al. 2011). The most distinct, both geographically and genetically, is the Goldman's Warbler (*Setophaga goldmani*) in Guatemala (Figure 1; Milá et al. 2008, 2011, Brelsford et al. 2011). The Goldman's Warbler is larger than the other taxa in the system, has darker plumage and unique white patches on the crown and sides of the throat, and is sedentary year-round (Milá et al. 2008). Also in Central America, the Black-fronted Warbler (*S. auduboni nigrifrons*) occurs in the mountains of northern Mexico and is also presumed to be sedentary (Milá et al. 2008, Toews et al. 2014b). In North America, Audubon's Warbler (*S. auduboni auduboni*) is found across much of Western North America and is currently considered conspecific with the Black-fronted Warbler by all the official nomenclature committees (although it was originally described as distinct species; Bent 1953). Audubon's Warbler shows a pattern of genetic intermediacy in amplified fragment length polymorphism (AFLP; Brelsford et al. 2011) between Black-fronted and Myrtle warblers, and there is a south to north gradient in both genetic and phenotypic traits across Audubon's Warbler. Finally, the Myrtle Warbler occurs from eastern North America west across most of Canada and into Alaska, hybridizing with the Audubon's Warbler in a narrow hybrid zone in British Columbia (Hubbard 1969, Brelsford and Irwin 2009).

Previous phylogeographic studies reveal a complex history of isolation and secondary contact. For instance,

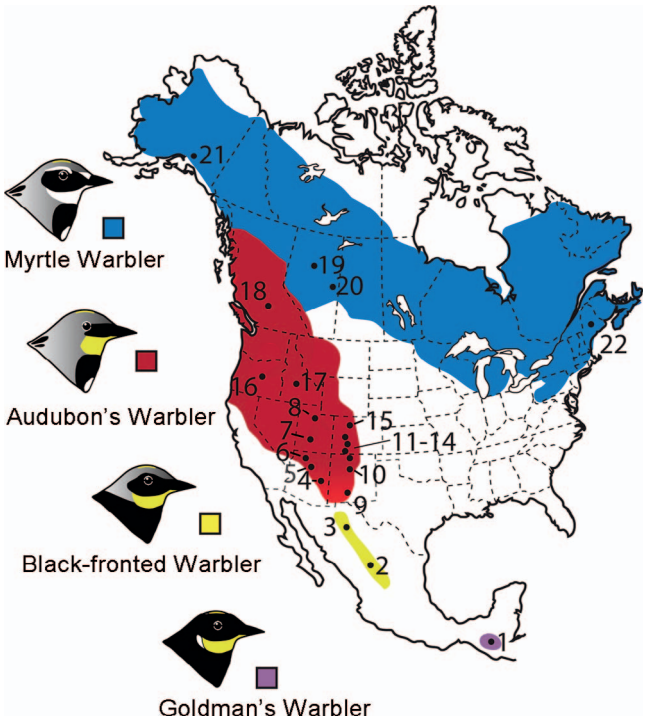

FIGURE 1. Distribution of breeding ranges for the Yellow-rumped Warbler (*Setophaga* spp.) complex. Taxa include Myrtle Warbler (*S. coronata*; blue); Audubon's Warbler (*S. auduboni auduboni*; red); Black-fronted Warbler (*S. a. nigrifrons*; yellow); and Goldman's Warbler (*S. goldmani*; violet). See Table 1 for additional information on sampling sites.

the Myrtle–Audubon warbler hybrid zone has been subjected to a number of detailed previous studies suggesting that hybrids form frequently, and there is little evidence of assortative mating (Hubbard 1969, Brelsford and Irwin 2009, Toews et al. 2014a). The hybrid zone is narrow, however, and is likely maintained by some form of selection against hybrids, indicating at least partial reproductive isolation between the 2 taxa (Brelsford and Irwin 2009). The Audubon's Warbler itself seems to have a mixture of genetic and phenotypic traits of Black-fronted and Myrtle warblers, suggesting either recent divergence or that hybridization has played some role in shaping this group (Brelsford et al. 2011). By contrast, there is no evidence of range overlap or hybridization between Goldman's Warbler with any of the other taxa in the group. Finally, although some historical records exist of putative hybrids between Black-fronted and Audubon's warblers (e.g., Bent 1953), the 2 taxa are not known to come into contact in the small sky islands that separate their breeding ranges and are effectively allopatric. Marked phenotypic differentiation, despite widespread sympatry during the nonbreeding season in Western Mexico, is consistent with reproductive isolation between the 2 forms (Milá et al. 2007).

Drawing from populations sampled across this spectrum of divergence, we employed a genotyping-by-sequencing (GBS) method (Elshire et al. 2011, with modifications by Alcaide et al. 2014) to generate a large genomic dataset to quantify genomic patterns of divergence. We first described patterns of genetic structure across the groups to better understand patterns of divergence across the complex and then compared how this divergence varied across the genomes of each of the pairwise comparisons. In some cases we found evidence of a highly heterogeneous pattern of differentiation. We were then interested in understanding the evolutionary processes that may have influenced this heterogeneity. We first tested whether the small divergent regions were enriched for genes, an important substrate for possible selection, and whether particular classes of genes were over-represented. We then compared the level of diversity, as measured by heterozygosity, for markers within and outside these divergent regions to test for evidence of reduced variation, possibly due to selection. We also employed a more formal test of selection, using a Bayesian outlier approach.

METHODS

Sampling

We sampled 94 Yellow-rumped Warblers (Myrtle, $n = 18$; Audubon's, $n = 57$; Black-fronted, $n = 14$; and Goldman's, $n = 5$) during the breeding season using song playback and mist nets (Table 1). Samples were obtained between 2001 and 2006 by B. M. in Guatemala (site 1), Mexico (sites 2 and 3), Idaho (site 17), and Maine (site 22), some of which were included in the AFLP assay of this system by Brelsford et al. (2011). Additional samples were collected by A. B. and D. I. from Gavin Lake (site 18), Slave Lake (site 19), and Cold Lake (site 20) in 2005, and from Anchorage (site 21) in 2007. Samples from Oregon (site 16) were obtained from the Burke Museum. In 2010 and 2011, D. P. L. T. collected many samples of Audubon's Warbler (sites 4–15).

Blood samples, taken using a small needle and capillary tube from the brachial vein, were stored in Queen's lysis buffer (Seutin et al. 1991) and left at ambient temperature until returned to the laboratory for analysis of genotypes. DNA was extracted using a phenol-chloroform protocol and resuspended with 50–200 μL of buffer (depending on the size of the pellet) containing 10 mM Tris-HCl and 1 mM EDTA at pH 8.0 and stored at 4°C.

Molecular Analysis

To generate genomic data, we used a reduced complexity GBS method (Elshire et al. 2011) adapted for use in our laboratory (Alcaide et al. 2014). We first standardized the concentration of all of the DNA samples to 20 ng μL^{-1}. From each diluted sample we then took 5 μL to add to a

TABLE 1. Sampling localities, sample sizes, and taxa sampled throughout the range of the Yellow-rumped Warbler complex.

Site	Average latitude	Average longitude	*n*	Species
1) Guatemala	15.31	−90.11	5	Goldman's
2) Durango, Mexico	24.54	−104.60	3	Black-fronted
3) Chihuahua, Mexico	28.61	−106.06	11	Black-fronted
4) Apache National Forest, AZ	33.99	−109.43	1	Audubon's
5) Coconino National Forest, AZ	35.15	−111.56	4	Audubon's
6) Kaibab National Forest, AZ	36.67	−112.20	4	Audubon's
7) Fish Lake National Forest, UT	38.61	−111.65	5	Audubon's
8) Uinta National Forest, UT	40.49	−111.63	4	Audubon's
9) Lincoln National Forest, NM	32.96	−105.74	3	Audubon's
10) Santa Fe National Forest, NM	35.89	−106.63	3	Audubon's
11) Carson National Forest, NM	36.70	−106.23	3	Audubon's
12) Rio Grande National Forest – Stunner, CO	37.38	−106.60	3	Audubon's
13) Rio Grande National Forest – Poso, CO	37.97	−106.53	5	Audubon's
14) Gunnison National Forest, CO	38.86	−106.72	5	Audubon's
15) Arapaho National Forest, CO	40.29	−106.05	5	Audubon's
16) Oregon	44.11	−120.16	4	Audubon's
17) Idaho	43.91	−114.94	3	Audubon's
18) Gavin Lake, BC	52.49	−121.71	5	Audubon's
19) Slave Lake, AB	55.49	−114.85	2	Myrtle
20) Cold Lake, AB	54.74	−110.07	4	Myrtle
21) Anchorage, AK	61.16	−149.72	7	Myrtle
22) Maine	45.16	−69.34	5	Myrtle

digestion mixture that included 6 μL of common adaptors (0.4 ng μL^{-1}) and 6 μL of barcoded adaptors (0.4 ng μL^{-1}; see Appendix Table 3 for barcode sequences), 20 units of the high fidelity PstI restriction enzyme (New England Biolabs), and 2 μL of the provided buffer (10X). The barcodes were variable in length, 4–8 bp, and every pair differed by at least 3 nucleotides; we used these to identify each individual later in the analysis. The resulting mixture was then incubated at 37°C for 2 hours. Following the digestion, to each sample we added 640 units of T4 DNA ligase (New England Biolabs), with 5 μL of the provided buffer (10X) and 23.4 μL of UltraPure water. We incubated this ligation reaction for 1 hr at 22°C and then inactivated the enzyme by incubating the mixture at 65°C for 10 min.

We cleaned this reaction using AMPure XP beads (Beckman-Coulter) to remove unused enzyme and small DNA fragments. In a new plate, we added 15 μL of the ligation mixture to 23 μL of beads and mixed thoroughly with a pipette. The samples were placed onto a magnetic plate and washed twice with 200 μL of 70% ethanol. The beads were then removed from the magnetic plate and resuspended in 40 μL of 1X TE. The samples were again placed on the magnetic plate, and the solution was removed and added to a new plate. We then performed a polymerase chain reaction (PCR) for each sample separately. Each 25 μL reaction was prepared on ice and included 0.5 units of PhusionTaq (New England Biolabs), 5 μL of 5X Phusion Buffer, 0.5 μL of 10 μM dNTPs, 0.125 μL of forward and reverse GBS primers (200 μM; see Elshire et al. 2011 for sequences), 18 μL of UltraPure water, and 1 μL of the cleaned DNA fragments from the ligation reaction. For the PCR, we used a thermocycling profile of 98°C for 30 s followed by 20 cycles at 98°C for 10 s, 65°C for 30 s, and 72°C for 30 s. This profile was followed by an extension at 72°C for 5 min. We quantified the product of this amplification and visualized it on a 2.5% agarose gel. Each sample was then added to a pool, and 25 μL was run in one of 3 lanes of a 2% agarose gel. We used a gel extraction kit (Qiagen) to isolate the final libraries within a size range of 300–400 bp, confirmed using a high sensitivity Bioanalyzer chip (Agilent Technologies) and quantified using qPCR. The final libraries were sequenced using paired-ends on an Illumina HiSeq 2000.

Demultiplexing and Adapter Removal

We demultiplexed sequencing reads using the barcode-splitting program Sabre (https://github.com/najoshi/sabre), allowing one mismatch in the barcode + enzyme cut-site sequence (the variable length barcodes we used differed by a minimum of 3 bp). We then used AdapterRemoval (1.5.4; Lindgreen 2012) to collapse paired reads with evidence of overlap between the pairs. This type of overlap occurs in paired-end sequencing when the DNA insert size is small and the same region is sequenced from both ends (also known as "read through"; Lindgreen 2012). It is important to collapse "read through" for genotype calling later in the analysis, where separate reads from the same DNA molecule should not be considered as independent. Paired reads that had no overlap were kept separate.

Alignment and SNP Calling

We used BOWTIE2 (2.1; Langmead and Salzberg 2012) to map each of the individual reads to a build of the Zebra Finch (*Taeniopygia guttata*) genome (Warren et al. 2010). For this we used the "very sensitive local" set of alignment presets. For single nucleotide polymorphism (SNP) discovery and variant calling, we used the UnifiedGenotyper in GATK (DePristo et al. 2011) and followed the Van der Auwera et al. (2013) set of GATK "best practices" as a guideline. Importantly, we removed possible variants that had "quality by depth" (QD) of <2 and "mapping quality" (MQ) of <30 (the full filtering expression we employed was: QD < 2.0, Fisher's exact test of strand bias > 40.0, MQ < 30.0, HaplotypeScore > 12.0, MappingQualityRankSum < -12.5, ReadPosRankSum < -8.0). Variant confidence is a measure of sequencing depth at a given variant site; mapping quality refers to the root-mean-square of the mapping confidence (from BOWTIE2) of reads across all samples.

Additional Filtering and Population Genetic Analysis

We applied additional filters using the program VCFtools (Danecek et al. 2011). First, we coded genotypes with a Phred-scaled quality <20 as missing data, which corresponds to a genotyping accuracy of at least 99%. Then we excluded loci with $>40\%$ missing data and/or a minor allele frequency of $<4\%$. To visualize the data and test for population structure, we used a principal components analysis (Patterson et al. 2006) using the SNPRelate package (Zheng et al. 2012) in R (R Development Core Team 2013). We retained the number of eigenvectors for which there were significant differences using an ANOVA in R, using the 4 taxonomic groups as distinct states within a categorical variable. Patterson et al. (2006) suggest the Tracy-Widom distribution may be the most appropriate distribution to test the significance of eigenvectors, although in practice the *F* distribution used in the ANOVA produces similar results (Patterson et al. 2006), which we used here.

High F_{ST} Clustering Analysis

To compare patterns of divergence across the genomes between each of the 4 groups, we used VCFtools (Danecek et al. 2011) to estimate F_{ST} (Weir and Cockerham 1984) for each locus between each of the groups (i.e. 6 comparisons). We tested for clustering of high F_{ST} loci between the genomes of these groups using the method described by Renaut et al. (2013). In brief, for each comparison we identified the loci within the top 1% of the distribution of F_{ST} estimates. Using a simple quantile to estimate outliers allows a robust comparison among groups that vary in their genome-wide distribution of divergence (Renaut et al. 2013). We then used a window across the genome and counted the number of high F_{ST} markers within each. We explored a variety of window sizes (i.e. 500 Kb to 2 Mb) and found that the results were qualitatively not sensitive to varying this parameter within that range; hence, we only report results from implementing a window size of 1 Mb (for scale, the longest chromosome in the Zebra Finch, chromosome 2, is $\sim$156 Mb). To test whether clusters of highly differentiated markers could be due to chance sampling, we used a permutation test. For each window we counted the number of total markers, randomly sampled that same number of markers from across the genome, and then counted the proportion of markers in the random sample identified as within the genome-wide top 1% threshold of F_{ST} estimates. Within each window, we conducted 1,000 permutations and defined statistical significance if the observed proportion was $>99\%$ of the observations from the random permutations (i.e. a critical value of 0.01). This procedure allowed us to objectively quantify the number of clusters with high F_{ST} between each of these comparisons. Note that this method will become less sensitive as genome-wide divergence increases; as background divergence becomes high, the ability to detect clusters of highly differentiated loci will diminish.

We calculated a coarse-scale size of each cluster by counting consecutive 1 Mb windows that showed significant clustering from the permutation test. For instance, clustering observed in 2 consecutive windows would equal a contiguous cluster size of 2 Mb. We then calculated the average cluster size in a given comparison (i.e. a genome with one 1 Mb cluster and two 2 Mb clusters would have an overall average cluster size of 1.7 Mb). For each of the comparisons we also calculated the observed heterozygosity for markers within and outside the divergence cluster using VCFtools.

Characterizing the Genomic Elements in Divergence Peaks between Myrtle and Audubon's Warblers

When comparing patterns of divergence between Myrtle and Audubon's warblers, we found evidence for a number of high-F_{ST} clusters (see results). This comparison had our highest sample size and therefore allowed us to perform a more detailed analysis. We examined whether these highly differentiated regions occur in gene-rich regions of the genome, presenting the most likely substrate for selection. For this analysis, we used the BioMart package within the Bioconductor environment (Kasprzyk 2011). We first estimated the number of genes annotated in the Zebra Finch within each 1 Mb window where we found evidence of significant clustering of differentiated markers between Myrtle and Audubon's warblers. Because mapping reads in coding regions will be more efficient than in noncoding regions (presumably because exons are more conserved across diverged taxa) and because the PstI enzyme cuts more frequently in gene-rich regions (Alcaide et al. 2014), we also used a permutation test to control for a correlation

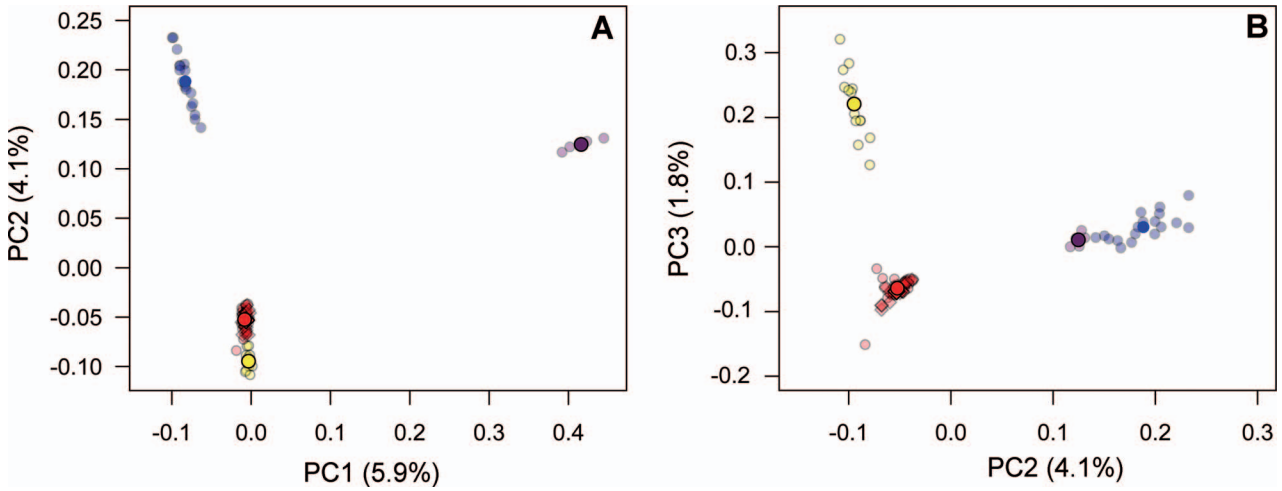

FIGURE 2. Principal components analysis (PCA) based on 37,518 polymorphic SNPs. The colors represent the 4 groupings presented in Figure 1. Transparent points show individual values, and opaque circles show the average value for each group. A PCA excluding Goldman's warblers is shown in Appendix Figure 9.

between gene number and marker number. We calculated the number of windows that showed significant clustering between Myrtle and Audubon's warblers and then randomly sampled the Zebra Finch for this number of windows and estimated, for each sample, the expected number of genes and markers. We ran this for 10,000 iterations to generate a null distribution and compared the result to the number of genes vs. markers in our sample. We also tested for significant evidence of enrichment of gene ontology terms (GO terms) in these windows with significant clustering by using all the annotated genes in these divergent regions and the GO analysis website for the Zebra Finch (http://www.ark-genomics.org/tools/GOfinch; Wu and Watson 2009). We tested for possible selection across all the loci in this comparison by using the program BayeScan (2.1; Foll and Gaggiotti 2008), which performs a locus-by-locus analysis of allele frequency variation and compares models of this variation where selection is or is not implicated. From these models, the program estimates the probability of selection acting on that locus and, after applying a false discovery rate (FDR; 0.05 in this case), determines a set of outliers.

RESULTS

Patterns of Genomic Divergence Across the Yellow-rumped Warbler Complex

Sequencing resulted in 238 million reads, a mix of paired and collapsed single reads (depending on AdapterRemoval paired read collapsing), and, following demultiplexing, an average of ~2.5 million reads per individual (Appendix Figure 7A). The average overall alignment rate of reads to the Zebra Finch reference across all samples was 56%, consistent across individuals and the different taxonomic groups (Appendix Figure 7B). Following filtering, we identified 37,518 polymorphic SNPs associated with known locations in the Zebra Finch genome. The principle component analysis (PCA; Figure 2) revealed strong evidence of genetic differentiation among all taxa. The ANOVA identified PC1, PC2, and PC3 to be highly significant when considering the *a priori* grouping of individuals as categorical variables in the model (PC1: $P < 0.001$; PC2: $P < 0.001$; PC3: $P < 0.001$), whereas PC4 was not (PC4: $P = 0.91$). This finding was confirmed by visual inspection of the cumulative explained variance; little additional variance was explained with PC4 (Appendix Figure 8). PC1 and PC2 (collectively explaining 10.0% of the variation) split individuals into a number of distinct clusters. Goldman's Warbler was separated strongly from the others along PC1. Myrtle, Audubon's, and Black-fronted warblers separated along PC2 (Figure 2). PC3, explaining 1.8% of the variation, separated Audubon's Warbler from the Black-fronted Warbler (Figure 2; see Appendix Figure 9 for the PCA without Goldman's Warbler included).

The divergence noted in the PCA translates into variable levels of relative differentiation between the groups, as measured by F_{ST} (Figure 3). Comparisons with Goldman's Warbler showed the highest level of differentiation across all of the comparisons, with mean weighted pairwise F_{ST} estimates ranging from ~0.18 to 0.26 (Figure 4). The next most divergent group of comparisons involved those with the Myrtle Warbler. Pairwise comparisons of Myrtle Warbler with Audubon's and Black-fronted warbler populations had mean weighted F_{ST} estimates of 0.06 and 0.08, respectively. The PCA clearly separates Audubon's and Black-fronted warblers along PC3, yet we found evidence of low levels of genetic differentiation between these groups (mean weighted $F_{ST} = 0.02$; Figure 4). Note, however, that our sample of Audubon's Warbler includes

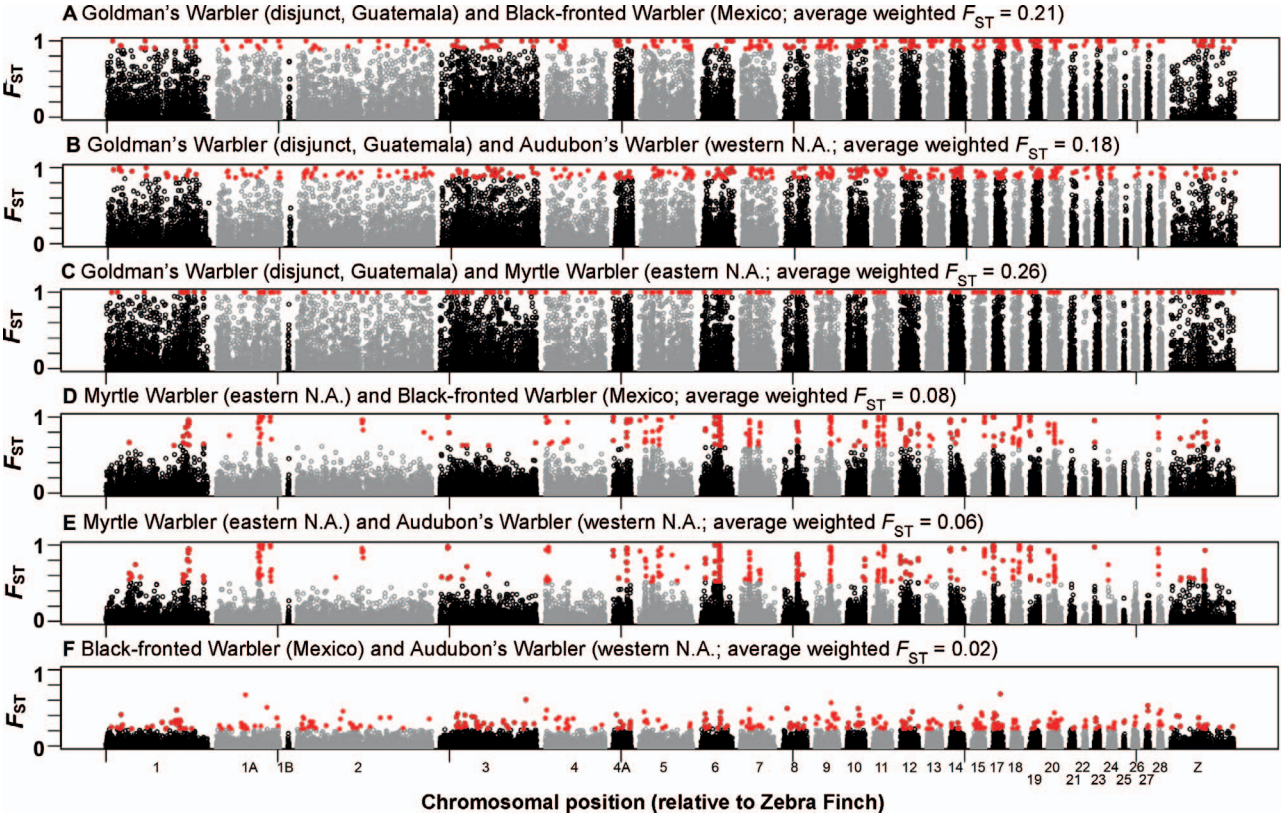

FIGURE 3. F_{ST} estimates for each locus relative to its position in the Zebra Finch genome. Comparisons are grouped into 4 categories (Myrtle, Black-fronted, Goldman's, and Audubon's warblers). The top 1% of markers in each case is highlighted with red circles. N.A. = North America.

fewer individuals from the northern part of their range, where they are most divergent from the Black-fronted Warbler (Brelsford et al. 2011).

The spatial patterns of differentiation across the genome varied widely between the groups (Figure 3). For example, the comparisons between Goldman's Warbler and Black-fronted or Audubon's warblers (Figure 3A and B) showed high levels of relative differentiation across the genome, and almost all chromosomes had many SNPs that were fixed or nearly fixed for alternative alleles. By contrast, comparing Myrtle Warbler with Audubon's or Black-fronted warblers (Figure 3D-E) revealed a different pattern:

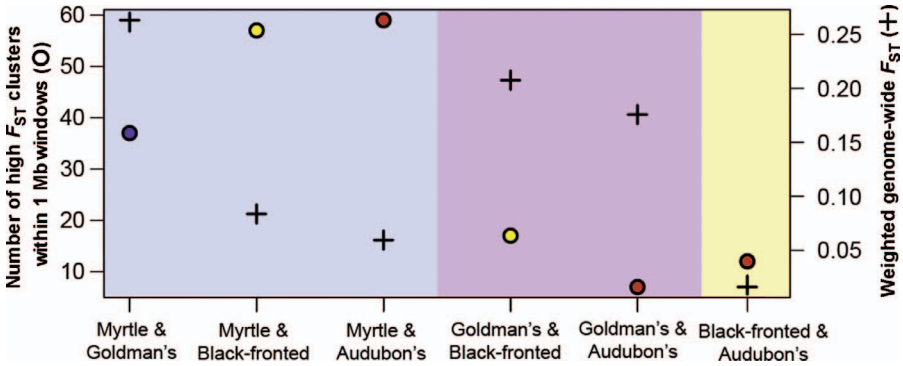

FIGURE 4. The number of clusters of highly differentiated markers and weighted genome-wide F_{ST} estimates between each of the 4 groups (Myrtle, Black-fronted, Goldman's, and Audubon's warblers). The number of clusters are shown in the circles, with the plot color and the point color corresponding to one of the groups in the comparison (i.e. the blue portion of the graph with a yellow circle compares Myrtle and Black-fronted warblers; see Figure 1 for color information). Clusters were estimated using 1 Mb windows following the procedure outlined by Renaut et al. (2013). Mean weighted F_{ST} estimates for each comparison are indicated by crosses.

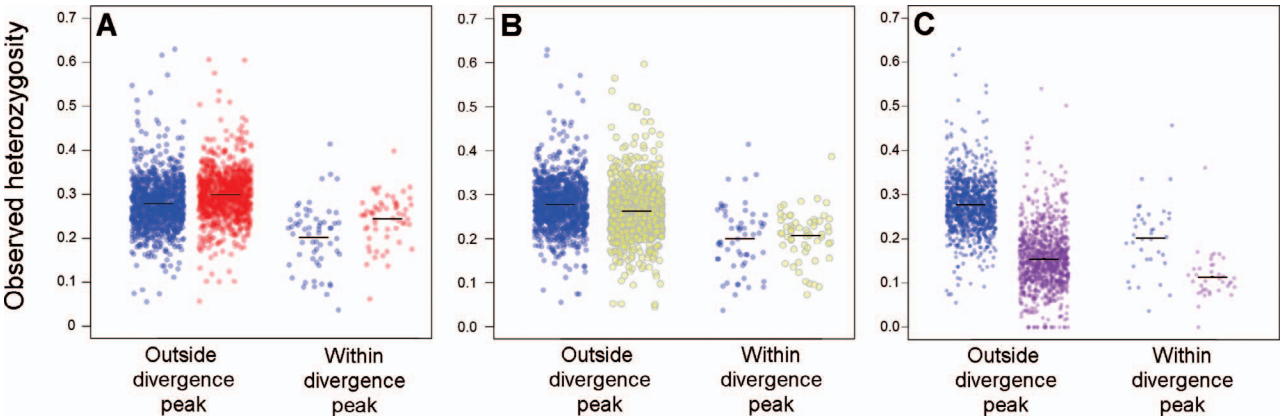

FIGURE 5. Estimates of observed heterozygosity across 1 Mb windows either outside or within a divergence cluster. Only the 3 comparisons with Myrtle warblers are shown because these had the strongest pattern of clustering (Figure 4). **(A)** Myrtle Warbler (blue) with Audubon's Warbler (red) comparison. **(B)** Myrtle Warbler (blue) with Black-fronted Warbler (yellow) comparison. **(C)** Myrtle Warbler (blue) with Goldman's Warbler (purple) comparison.

clusters of highly differentiated regions, separated by regions of low average F_{ST}, scattered in distinct areas. For example, between Audubon's and Myrtle warblers, chromosome 3 showed little evidence of high F_{ST} clusters in these comparisons, whereas chromosome 9 had a clear high divergence peak (see Appendix Figure 10 for closer view of an example chromosome). Much less differentiation was observed in the comparison of Black-fronted and Audubon's warblers (Figure 3F), in which only 7 markers have F_{ST} values >0.5.

Our permutation test was designed to objectively identify the extent of clustering of high F_{ST} markers in discrete windows (Figure 4). In agreement with the qualitative patterns from the Manhattan plots (Figure 3), comparisons with the Myrtle Warbler showed more evidence of spatial clustering of highly differentiated markers (i.e. 37–59 clusters). The highest number of windows with significant clustering occurred between Myrtle and Audubon's warblers, with 59 windows of 1029 across the genome showing significant clustering. Each of these 1 Mb clusters contained, on average, 91 SNPs, and all but one had >10 SNPs. A number of significant regions also occurred with high F_{ST} clusters when comparing Myrtle and Goldman's warblers, which were less obvious in the Manhattan plot where many markers have high F_{ST} values (Figure 3). High F_{ST} clusters between Myrtle and Goldman's warblers occur in a similar genomic location as those between Myrtle and Audubon's warblers; 76% of the 37 clusters between Myrtle and Goldman's warblers also show significant clustering between Myrtle and Audubon's warblers. The other comparisons, not including the Myrtle Warbler, showed far fewer clusters (i.e. 7–17 clusters), similar to the expected false-positive rate (i.e. 1% of the 1029 windows). The comparisons with the Myrtle Warbler also had the largest estimated size of clusters, with the average size $\geq$2 Mb.

For comparisons with many divergence clusters (i.e. comparisons with the Myrtle Warbler; Figure 4), we found that average observed heterozygosity of markers was lower inside divergence clusters compared to outside (Figure 5). This finding was true for each of the taxa within a given comparison; these highly clustered F_{ST} regions were associated with reduced variation, as measured by heterozygosity, across all of the taxa. We also found that Goldman's Warbler had a lower genome-wide level of heterozygosity compared to the other taxa in the complex (Figure 5C).

Molecular Signatures of Selection between Myrtle and Audubon's Warblers

Focusing on the comparison between Myrtle and Audubon's warblers, the BayeScan analysis suggested that highly divergent loci between these taxa also showed evidence of selection (Appendix Figure 11). BayeScan identified 398 outlier loci when using an FDR of 5%; using a much more stringent FDR (e.g., 0.01%) still resulted in 190 outliers. There is also much overlap between these outliers and the top 1% of markers identified during the clustering analysis; 79% of markers in the top 1% were also identified as outliers by BayeScan (with the FDR set at 5%).

The BioMart analysis suggests that the high-cluster windows between Myrtle and Audubon's warblers tend to occur in gene-rich regions (Figure 6). Although these windows also had a large number of variant sites (correlated with overall gene number), these differentiated clusters seemed to have more genes than expected based on this relationship alone (Figure 6). For the 704 annotated genes that occur in these highly divergent regions, however, we found no evidence they were enriched for

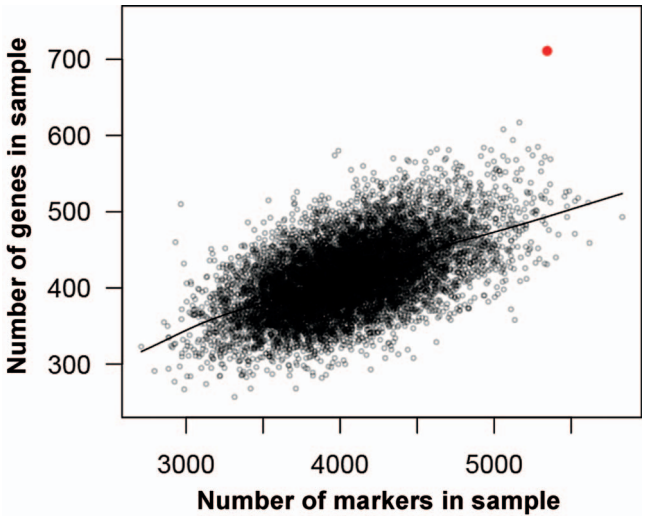

FIGURE 6. The number of annotated genes, when aligned with the Zebra Finch, within 1 Mb regions of the genome that show evidence of high F_{ST} clustering between Myrtle and Audubon's warblers (red point), estimated with BioConductor. The black points show 10,000 permutations where the same numbers of 1 Mb regions were sampled at random throughout the Zebra Finch genome. For each sample, the number of markers and genes within those regions are shown. The 2 have a positive relationship, although the sample between Myrtle and Audubon's warblers appears highly enriched for more genes than expected by chance alone.

any GO terms (the lowest adjusted Fisher test, $P = 0.24$) compared to the rest of the genome.

DISCUSSION

This work provides a reduced-representation genomic study of the Yellow-rumped Warbler sampled across its breeding range. These data complement previous studies in this system by allowing us to quantify, at a high resolution, levels of divergence across groups within this species complex and discover how this divergence varies across the genome. Compared with each of the other taxa, Goldman's Warbler shows high levels of differentiation across the genome, confirming patterns of previous studies using fewer genetic markers (Milá et al. 2007, Brelsford et al. 2011, Milá et al. 2011). This differentiation is presumably due to long-term isolation and current (and likely historic) small population size within a restricted geographic range (Milá et al. 2007, Brelsford et al. 2011). Under this scenario, genetic drift is predicted to result in high levels of differentiation between populations. The observation of reduced variation in this taxon, as measured by genome-wide heterozygosity, is consistent with the demographic effects of isolation and drift (Figure 5). One caution in interpreting these results is that our sample of Goldman's Warbler was not large, primarily because of the logistical difficulties involved with collecting samples in the remote region of Guatemala where these birds occur.

We found differences in the amount of genomic heterogeneity and levels of relative differentiation between the groups. For example, in comparisons between Goldman's–Audubon's warblers and Goldman's–Black-fronted warblers, the mean weighted genome-wide divergence estimates were high, whereas the number of high F_{ST} clusters was low (i.e. <20 clusters; Figure 4, Table 2). By contrast, we found many high F_{ST} clusters when comparing Myrtle with Audubon's, Black-fronted, or Goldman's warblers (i.e. >37 clusters across the genome for each of these comparisons; Figure 4, Table 2). This clustering is especially evident when comparing Myrtle and Audubon's warblers; their genomes show consistently low levels of differentiation punctuated by small regions of loci with high F_{ST} values (Figure 3D and E). Although in some cases

TABLE 2. Biogeographic patterns, history of hybridization, and estimates of genomic characteristics across taxonomic comparisons. To estimate the size of clusters, we averaged the number of consecutive 1 Mb windows that showed significant clustering from the permutation test (see methods). We then divided the result by the total number of clusters in a given comparison.

Taxa in comparison	Distribution and evidence of hybridization	Genome-wide weighted F_{ST} estimate	Number of high F_{ST} clusters	Average cluster size across consecutive 1Mb windows (Mb)
Goldman's–Myrtle	Allopatric – no evidence of hybridization	0.26	37	2.6
Goldman's–Audubon's	Allopatric – no evidence of hybridization	0.18	7	1.2
Goldman's–Black-fronted	Allopatric – no evidence of hybridization	0.21	17	1.6
Myrtle–Audubon's	Parapatric – extensive hybridization[1]	0.06	59	2.1
Myrtle–Black-fronted	Allopatric – no evidence of hybridization	0.08	57	2.0
Audubon's–Black-fronted	Allopatric – historical hybridization[2]	0.02	12	1.3

[1] Hubbard (1969), Brelsford and Irwin (2009)
[2] Bent (1953)

these clusters of high F_{ST} markers occur in neighboring windows, approximately half (31 of 59 windows) are noncontiguous and occur on a variety of chromosomes. Gene flow between Audubon's and Myrtle warblers has possibly made observing peaks easier than comparisons with Goldman's Warbler, which has a much higher level of background divergence. This may be one reason why we found fewer clusters between Myrtle and Goldman's warblers than the other comparisons with Myrtle Warblers. We therefore suggest that although the qualitative patterns of the clustering analyses are robust, the absolute number of divergent regions should be treated with caution because it depends partially on the window parameters.

We offer 2 explanations for more clusters of highly differentiated markers between the comparisons with the Myrtle Warbler and other groups (Figure 4). First, something unique may have occurred in the evolutionary history of the Myrtle Warbler, in contrast to the other groups, that has driven divergence within the via natural or sexual selection. For instance, the Myrtle Warbler is the only member of this group that occurs throughout the Boreal forest and has a breeding range that extends far north, including the Yukon and Alaska (Hunt and Flaspohler 1998). The Myrtle Warbler also exhibits longer seasonal migratory movements compared to Audubon's, Black-fronted, or Goldman's warblers, and no known Myrtle Warbler populations are nonmigratory, which is not true of the other 3 groups. The Myrtle Warbler also has the most unique plumage patterning of the complex, with a white throat that contrasts with the yellow throat feathers of the other members of the group. A second, and related, interpretation involves the more general evolutionary relationship among the 4 taxa in the complex. For instance, Goldman's Warbler is differentiated from all the groups based on patterns of allele frequencies in SNPs (this study) and AFLPs (Brelsford et al. 2011). Other genetic (e.g., intron and mtDNA) and phenotypic data, however, suggest that Goldman's Warbler is more closely related to Audubon's and Black-fronted warblers than any of those are to the Myrtle Warbler (Milá et al. 2007, Brelsford et al. 2011). The higher number of divergence clusters between the Myrtle Warbler and each of the other taxa may therefore simply result from longer divergence times.

In contrast to studies of genomic divergence between other avian pairs, such as the Pied Flycatcher and Collared Flycatcher (Burri et al. 2015) and Coastal Swainson's Thrush and Inland Swainson's Thrush (Ruegg et al. 2014, Delmore et al. 2015), the regions of divergence between the Yellow-rumped Warbler taxa are small. Burri et al. (2015) implicated linked background selection across regions of reduced recombination, which is thought to contribute to the large tracts of divergence between several independent flycatcher taxa. In this case, chromosomal regions of reduced recombination are presumed to amplify the effects of selection between groups over large genomic regions compared to a scenario in which recombination is uniform across the chromosome (Burri et al. 2015). These regions are therefore shielded from the homogenizing effects of gene flow by both selection and reduced recombination. As is the case for many other non-model avian taxa, little information exists on the recombination landscape in warblers. Therefore, our conclusions are tentative regarding how drift or selection may or may not interact with reduced recombination in generating the clusters of markers with elevated divergence. Based on the observed size of divergence peaks, however, we can say with some certainty (even with the resolution of GBS data) that in this system any effects of linked selection and reduced recombination on divergence do not extend much farther than 1–3 Mb across a chromosome.

Our data do provide some indirect evidence that, as in other studies, genomically localized natural selection may be contributing to elevated divergence between the warbler groups. For example, between Myrtle and Audubon's warblers, we found that regions of divergence occur in gene-rich regions (Figure 6, Appendix Figure 12). Moreover, although the GO analysis found no evidence of enrichment of any functional categories, many genetic markers in these regions were identified by BayeScan as outlier loci, consistent with the effects of localized selection (Appendix Figures 11, 13, and 14). In addition, the small genomic outlier regions between the Yellow-rumped Warbler groups bear qualitative similarities (at least in terms of their small size) to comparisons between the Hooded Crow and Carrion Crow (Poelstra et al. 2014) and between the Blue-winged Warbler and the Golden-winged Warbler (Toews et al. 2016b). In those systems, regions of divergence were small and restricted enough that particular candidate genes could be investigated for their possible connection to the phenotypic differences between the species pairs. For example, the large island of divergence between the crow species includes genes involved in pigment metabolism and visual perception (Poelstra et al. 2014). Given the large number of divergent regions between several of the Yellow-rumped Warbler taxa, such a fine-scaled analysis is not currently possible. These findings, however, set the stage for future admixture analyses in the various contact zones, particularly the Myrtle–Audubon's warbler hybrid zone, which would facilitate associating phenotypic differences, such as plumage characters, with the genetic regions that underlie them.

As in other hybridizing avian taxa, gene flow is presumably an important force in homogenizing the genomes across the Yellow-rumped Warbler complex. Without more robust demographic modeling, however, it is currently unclear whether the regions of low divergence

between these warbler groups are a result of shared ancestral variation or gene flow following hybridization and introgression. We know from studies of the contact zone between the Myrtle and Audubon's warblers that current, and presumably historical, hybridization occurs between these groups (Hubbard 1969, Barrowclough 1980, Brelsford and Irwin 2009; Table 2), and phylogeographic patterns are consistent with historical mtDNA introgression from Myrtle Warblers and into some Audubon's Warbler populations (e.g., Milá et al. 2007, 2011, Brelsford et al. 2011, Toews et al. 2014b). Gene flow between some of these groups may have facilitated observing the divergence peaks by reducing the background levels of divergence, although the extent of introgression is difficult to determine from the current data sampled from allopatric individuals. Clearly, performing additional genomic assays from birds across regions of sympatry will be beneficial. An important question motivating this analysis will be how these divergent regions relate to the reproductive barriers and/or phenotypic differences between the groups. In particular, how do genomic regions that have diverged between allopatric populations of Myrtle and Audubon's warblers vary across their hybrid zone? This line of investigation would allow us to test whether the divergent genomic regions we have currently identified show evidence of contemporary selection against hybrids (i.e. narrow clines across the hybrid zone compared to the genome-wide average; e.g., Taylor et al. 2014).

There is clearly much power in the genotyping-by-sequencing approach employed here, although there are some important considerations to note. For instance, undoubtedly numerous narrow peaks of genomic differentiation were not detected by the GBS analysis. The method is designed to represent only a fraction of the genome, an unavoidable cost associated with sequencing common genomic regions across many individuals in an affordable way (Elshire et al. 2011). However, it is notable that some previously identified markers known to be fixed for alternate alleles in Audubon's and myrtle warblers, including CHD1Z, were not represented in our survey (Brelsford and Irwin 2009). This implies that there are still likely more clusters of high differentiation that our current analysis did not have the resolution to detect. Given that many of the short sequencing reads could not be mapped directly to the Zebra Finch genome, we suggest that sequencing the full genome of a New World warbler, combined with population resequencing, will allow us to quantify divergence at a finer scale, estimate additional summary statistics, and compare levels of relative vs. absolute estimates of divergence (e.g., Delmore et al. 2015).

These new genomic data provide valuable information on the evolutionary history of the Yellow-rumped Warbler group and can be used to clarify some of the taxonomic boundaries among taxa. First, confirming the results of previous studies that relied on fewer genetic markers (Milá et al. 2007, Brelsford et al. 2011), the Goldman's Warbler is highly divergent throughout its genomes across all the comparisons, a pattern consistent with isolation of this group compared to the other taxa in this system. This, together with its strong phenotypic differentiation and geographic isolation, leads us to recommend that Goldman's Warbler be considered a distinct species. Second, although Myrtle and Audubon's warblers show less overall divergence compared to Goldman's Warbler, dozens of regions of their genomes are highly differentiated and contain fixed SNPs. These patterns suggest that, at least throughout a significant portion of their genomes, there is no longer gene flow, and, in combination with evidence of selection against hybrids in their hybrid zone (Brelsford et al. 2011), we recommend that Myrtle and Audubon's warblers should also be considered different species.

The proper taxonomic treatment of the Black-fronted Warbler compared to Audubon's Warbler is more debatable. Arguments for treating the Black-Fronted Warbler as a distinct species include: (1) it is phenotypically distinguishable from Audubon's Warbler (Hubbard 1969, Milá et al. 2008, 2011, Brelsford et al. 2011); (2) it is separated from Audubon's by a distributional gap (Figure 1); and (3) it is genomically differentiated from Audubon's Warbler (Figure 2B), with a distinct jump in genomic signature at the geographic boundary between them (Appendix Figures 15 and 16). However, the degree of phenotypic differentiation, is less pronounced than that found among the other taxa, the level of differentiation is very low ($F_{ST} = 0.02$) compared to other pairs of taxa in the complex (Table 2), and we identified no differentially fixed loci between them in our GBS survey. Note that our sampling focused mostly on the southern part of the Audubon's Warbler range, and previous work (Brelsford et al. 2011) indicates that inclusion of more northern Audubon's Warbler samples would likely lead to higher estimates of differentiation between Audubon's and Black-fronted warblers. In addition, as discussed earlier, our GBS analysis may have missed the most divergent parts of the genome between Audubon's and Black-fronted warblers. Given these observations, this analysis possibly underestimates the true amount of genomic differentiation between the 2 groups. We anticipate that the taxonomic treatment of the Black-fronted Warbler will continue to generate discussions among nomenclature committees. As a small-scale example of this debate, we note that even the authors of this current paper have differing opinions about whether *nigrifrons* and *auduboni* are best treated as 1 or 2 species. D.P.L.T., A.B., and C.G. prefer the recent status quo of one species until more data regarding reproductive isolation are obtained, whereas B.M. and D.E.I. believe the evidence

for at least partial reproductive isolation is sufficient and prefer the original treatment of the 2 taxa as distinct species.

In conclusion, we found a strong pattern of heterogeneous differentiation across a number of groups within this phenotypically diverse avian species complex. The accumulated evidence suggests that, in many cases, these divergence clusters are the result of selection. The more general pattern of genomic homogeneity among several of the groups in this system may be the result of gene flow following secondary contact, eroding genomic differences that may have evolved among them. Distinguishing gene flow from retained ancestral polymorphism is difficult, however, and therefore these and other questions should be addressed with additional studies of individuals sampled across hybrid zones between the taxa, whole genome sequencing, and demographic modeling. Understanding the relative role of different kinds of selection in shaping genomic variation, how these regions may or may not be linked to important phenotypic traits, and whether they influence levels of reproductive isolation are important avenues that additional genomic data can address.

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Author Contributions: D.P.L.T., D.E.I., A.B., and B.M. conceived the idea and design. D.P.L.T., A.B., and B.M. performed the experiments/collected samples. D.P.L.T., A.B., D.E.I., C.G., and B.M. wrote the paper. D.P.L.T. and C.G. developed or designed methods. D.P.L.T. analyzed the data.

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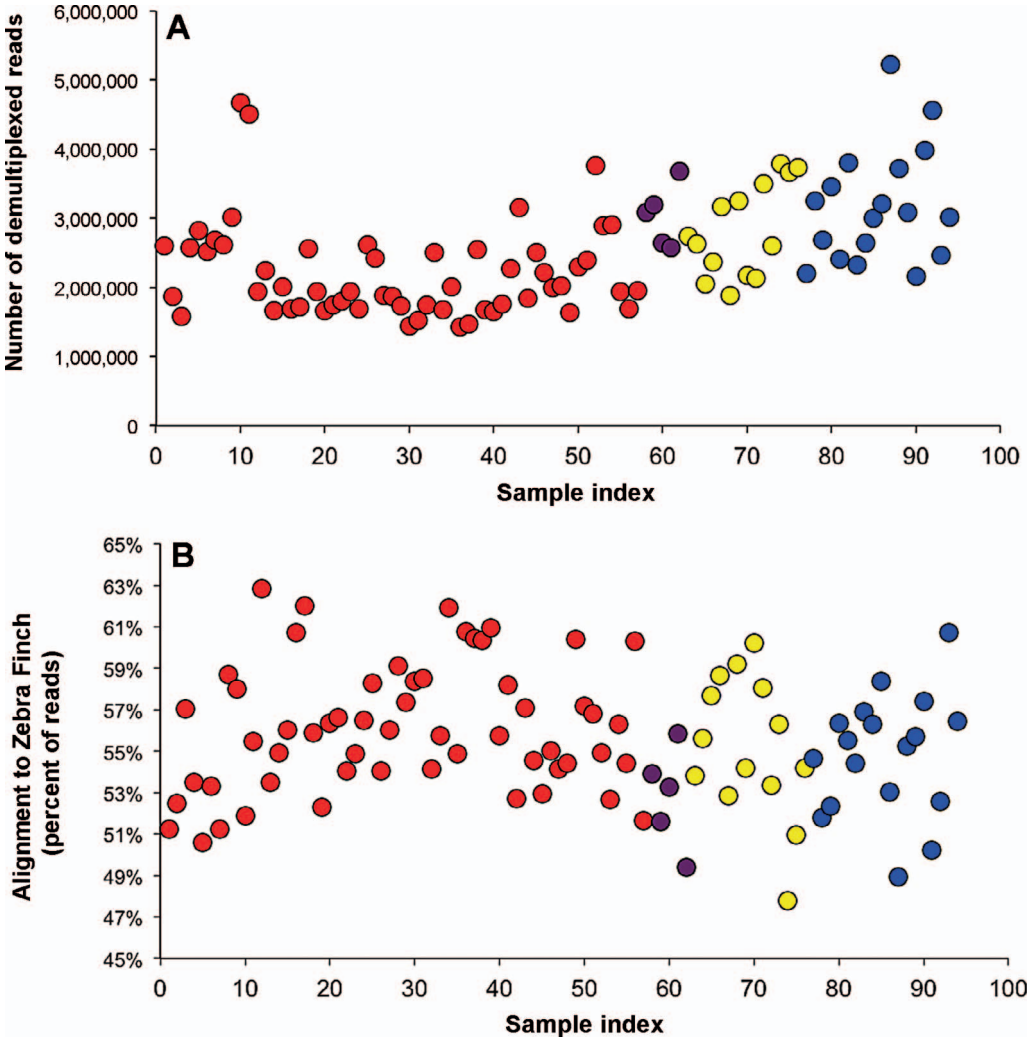
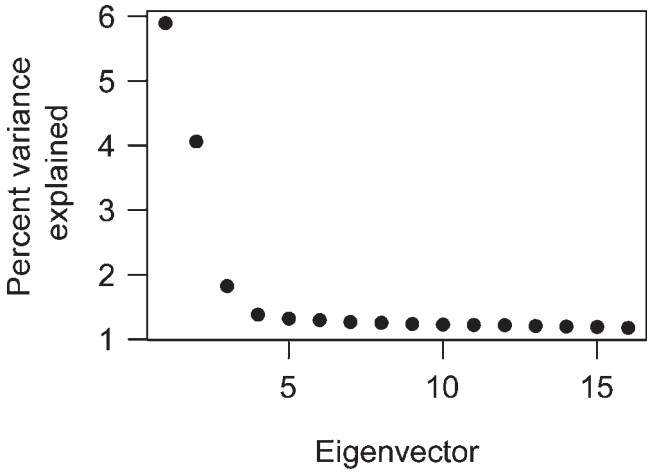
APPENDIX

APPENDIX TABLE 3. Barcode sequences used in the analysis.

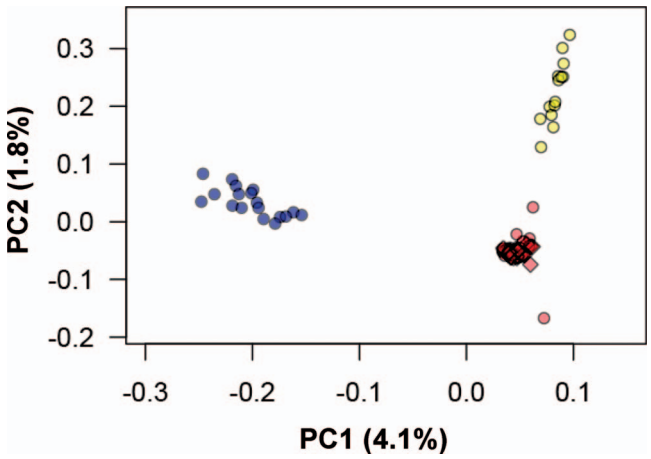
Number	Barcode sequence
1	ACGG
2	TGCT
3	CATA
4	CGAG
5	GCTT
6	ATCA
7	GACG
8	CTGT
9	TCAA
10	AGTCA
11	TCACG
12	CTGCA
13	CATCG
14	ATCGA
15	TCGAA
16	ACCTG
17	CTCAG
18	CGCTA
19	CCTGA
20	CGACT
21	ACGCT
22	GCCAT
23	CACGT
24	GTTCCA
25	TGTGCA
26	TTGACA
27	AGCTGA
28	TGGCAA
29	CTATCG
30	GCTGAA
31	TTCCGA
32	GACTCT
33	ATGGCG
34	TCATGG
35	CATCCG
36	CCGTCA
37	GTACGT
38	TAGGCT
39	GGCTAG
40	CATGTA
41	ATTCGG
42	TGACCT
43	GCTACT
44	TCGGTA
45	CTGAGG
46	GCCTTA
47	CGATGT
48	GATTACA
49	GGTAGCA
50	GTGACCA
51	TTATGCA
52	ATTGGCA
53	TGGTACA
54	GACCTCA
55	TGTGCCA
56	TAGACCG
57	GGATTCA
58	GATCCAA
59	CTGGACA
60	AGACTCG
61	AATTGCG
62	TCCAGGA
63	TCAGCAG
64	CAGTGCA
65	GTACCGA
66	TGTAACG
67	TACGATA
68	GTAAGCG
69	ATGCAAT
70	CCGGTAA
71	AGCTCCG
72	AATGGACA
73	AGAATGCA
74	GAATAGCA
75	ATGAGACA
76	TGCCACCA
77	ATAGAGCA
78	ACTCGCCA
79	TAGGAACA
80	GATACGAA
81	GCACCTCA
82	CACTGCCA
83	ACGATGAA
84	CGCACACT
85	AGTGACAA
86	CAAGTAGA
87	GCAAGAAT
88	ACCTACCG
89	CTACCACG
90	TAGAACGA
91	AGCAGTAA
92	GAACTGAA
93	ACTCCACG
94	GAAGACAT
95	CGGTATGT
96	TCCGCACA



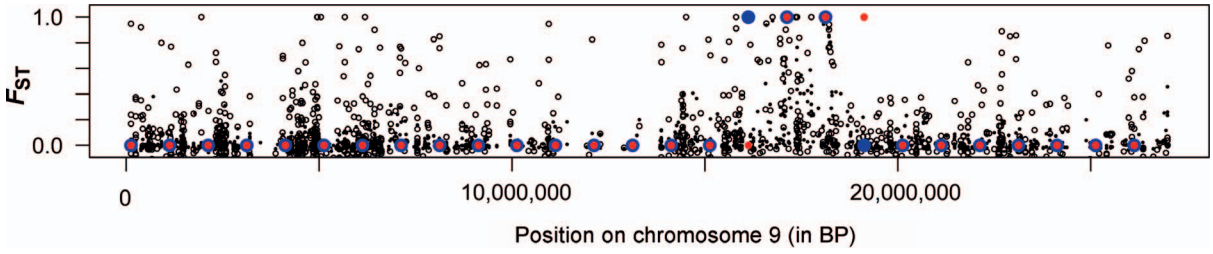
APPENDIX FIGURE 7. (**A**) The number of raw, unaligned reads assigned to each individual following demultiplexing, with the taxonomic groups indicated by different colors (red = Audubon's, purple = Goldman's, yellow = Black-fronted, blue = Myrtle). The average was 2,528,287 reads per individual. (**B**) The percentage of reads aligned of reads to the Zebra Finch reference for each warbler sample. The average assignment is 56% of the paired reads.



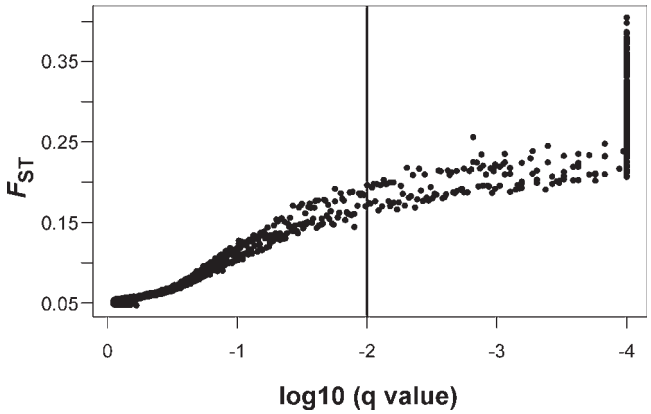
APPENDIX FIGURE 8. Percent of variance explained for each eigenvector for the principal components analysis with all samples included.



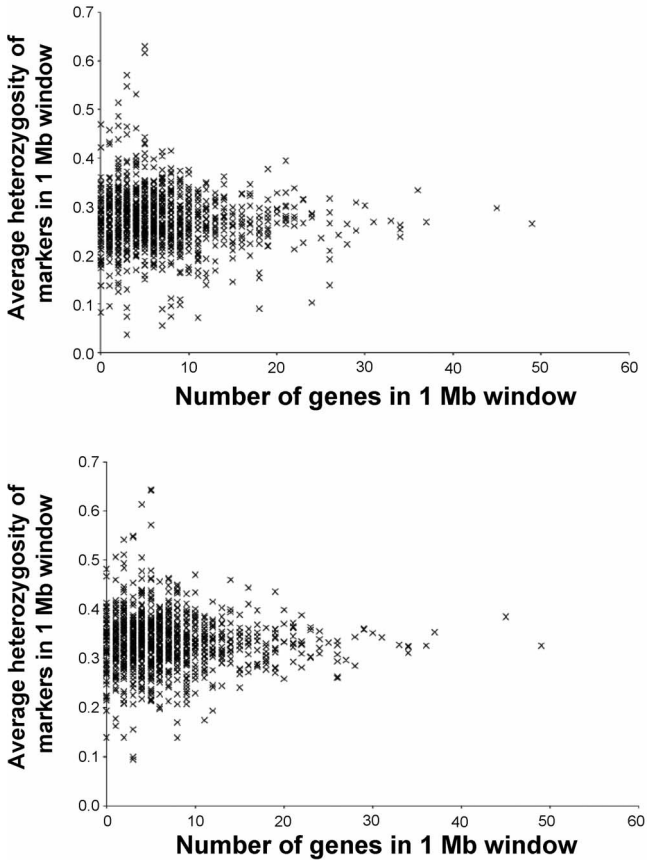
APPENDIX FIGURE 9. Principal components analysis (PCA) with Goldman's Warbler samples removed.



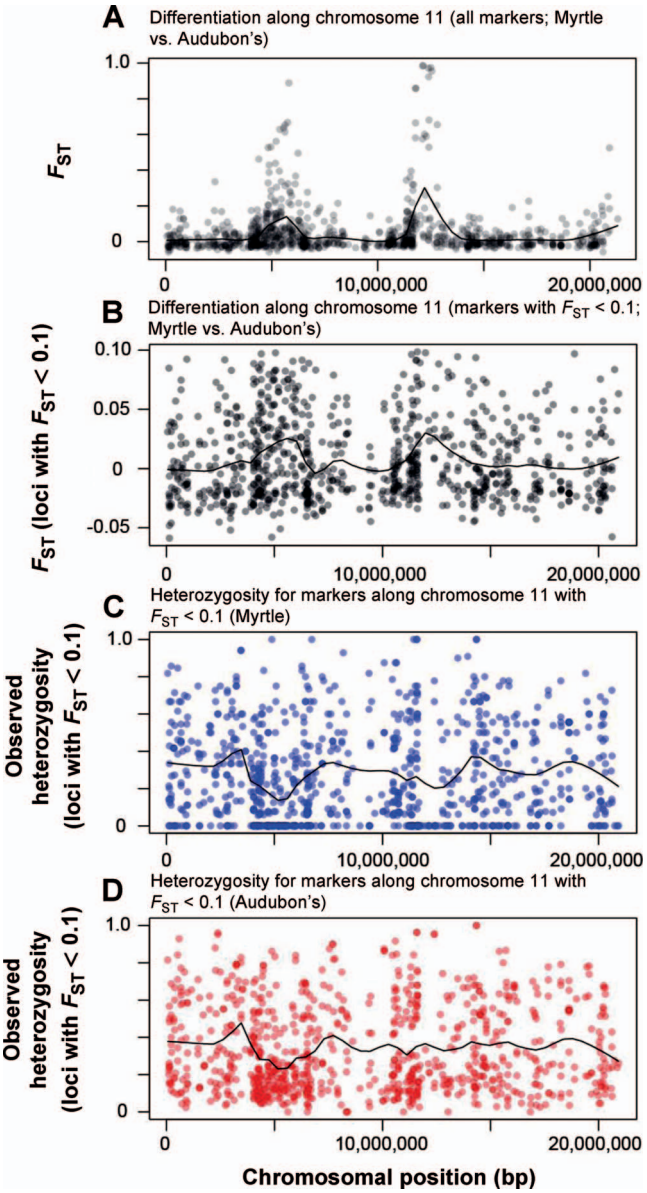
APPENDIX FIGURE 10. An example of high F_{ST} clustering along chromosome 9. The points show the F_{ST} value for a given SNP. Filled points are the values for the Myrtle–Audubon's comparison. The open points are the values for the Myrtle–Goldman's comparison. Colored circles represent the output from the high F_{ST} clustering analysis across 1 Mb windows. Binary values are 0 or 1, whether a window was found to have a significant number of high F_{ST} markers (see methods for information on the permutation test). Red circles indicate windows where the Myrtle–Audubon's warbler comparison had evidence of significant clusters; blue windows indicate where the Myrtle–Goldman's comparison had evidence of significant clustering.



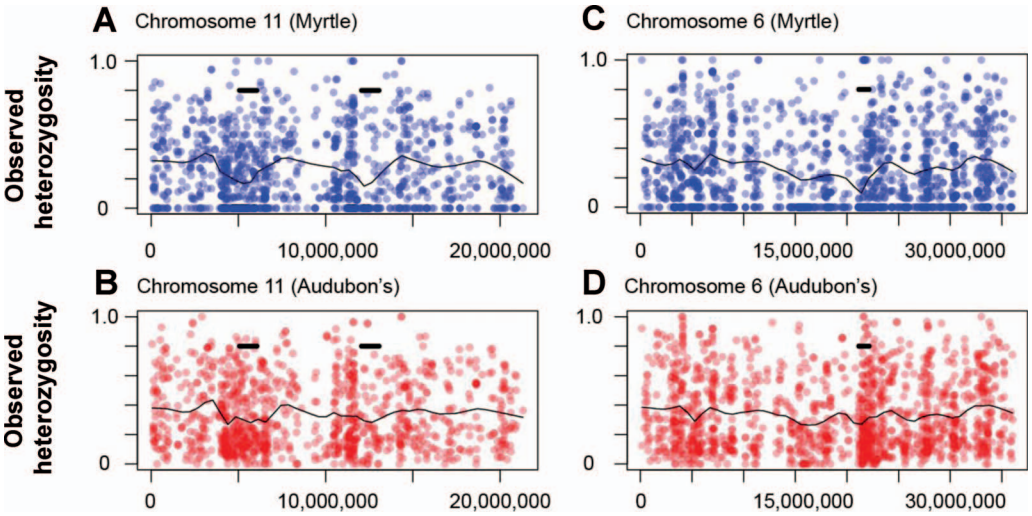
APPENDIX FIGURE 11. Output from the BayeScan analysis comparing Myrtle and Audubon's warblers. The vertical line shows the false discovery rate, here set at 0.01.



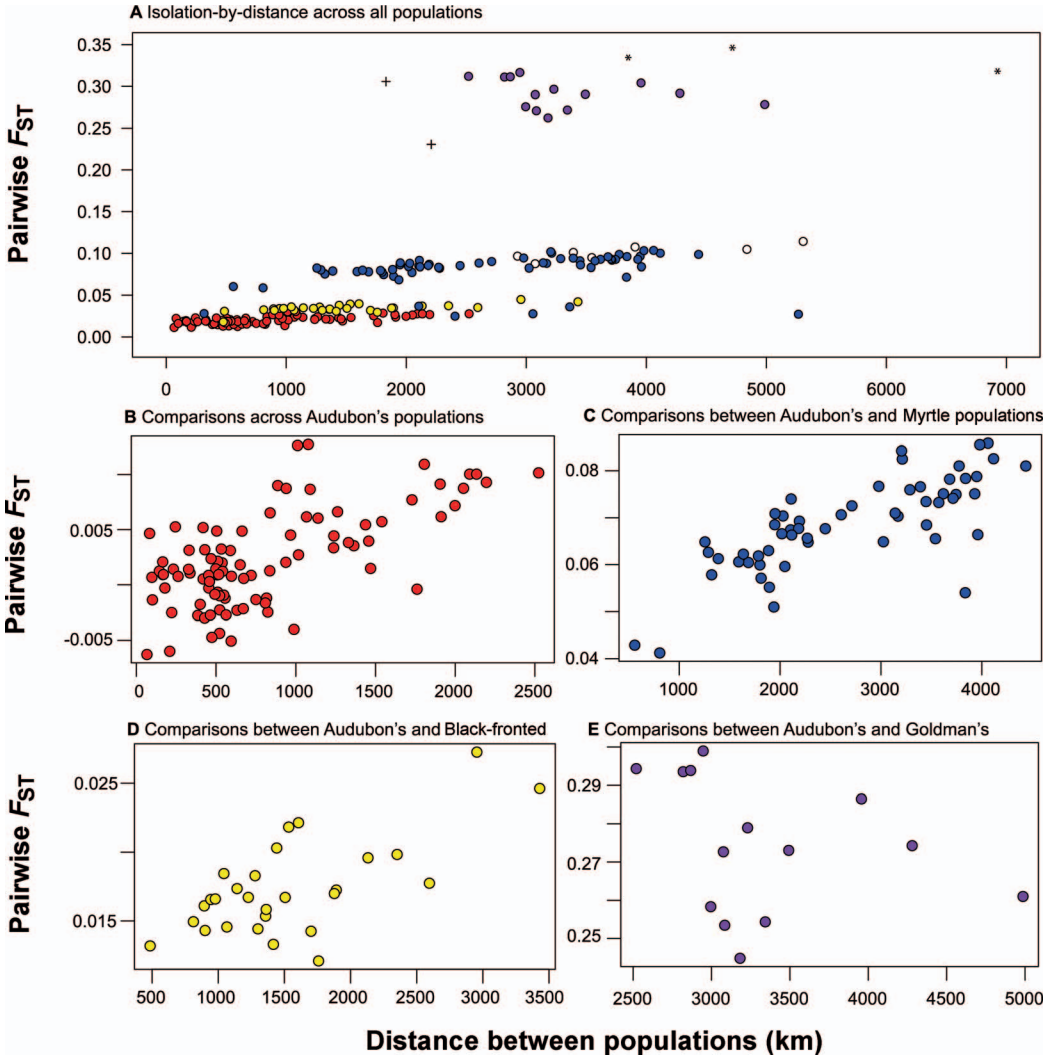
APPENDIX FIGURE 12. The relationship between gene number (as estimated from the Zebra Finch genome annotation) and average marker heterozygosity for 1 Mb windows for (**A**) Myrtle Warbler and (**B**) Audubon's Warbler.



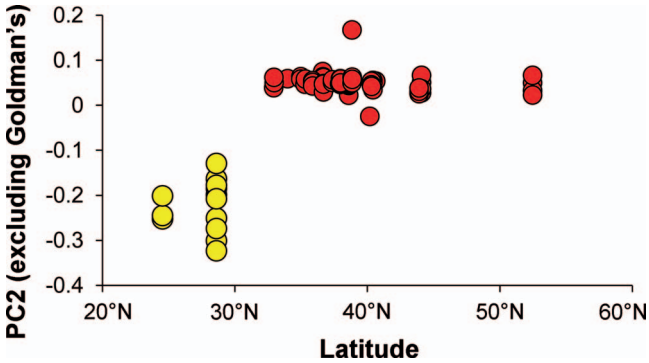
APPENDIX FIGURE 13. An example of chromosomal patterns of differentiation and observed heterozygosity in (**A** and **B**) Myrtle and Audubon's warblers (chromosome 11). Levels of heterozygosity are reduced in regions of high differentiation, even when the analysis is restricted to markers with low F_{ST} between (**C** and **D**) Myrtle and Audubon's warblers. The line shows a LOESS smoothing function, with the smoothing parameter (α) set to 0.12.



APPENDIX FIGURE 14. An example of chromosomal patterns observed for heterozygosity in Myrtle and Audubon's warblers: (**A** and **B**) chromosome 11 and (**C** and **D**) chromosome 6 are shown. The line shows a LOESS smoothing function, with the smoothing parameter (α) set to 0.12. The thick horizontal lines correspond to those regions with significant clusters of high F_{ST} markers (Figure 4). Both groups in the comparisons have, on average, lower heterozygosity in these regions, although the patterns are generally more accentuated in Myrtle Warbler (e.g., Appendix Figure 13C vs. 13D).



APPENDIX FIGURE 15. (A) The relationship between population pairwise F_{ST} estimates and the distance between all sampled populations (i.e. isolation-by-distance; IBD). Each point represents a comparison between 2 populations. Colored points represent comparisons between Audubon's Warbler and other Audubon's (red), Black-fronted (yellow), Myrtle (blue), and Goldman's (purple) warbler populations. Asterisks indicate the Myrtle–Goldman's comparisons; crosses indicate Black-fronted–Goldman's comparisons; open circles indicate Black-fronted–Myrtle comparisons the six low F_{ST} blue points also indicate comparisons within Myrtle warblers. Comparing Black-fronted and Audubon's warblers suggests some likely reduction in gene flow between these 2 taxa beyond geographic differences alone (i.e. yellow points indicate slightly higher genetic differentiation at comparable distances than red points), but, given the low levels of absolute differentiation, suggests any barrier between them is likely weak. (**B**–**E**) represent the same data as (**A**), but each panel shows a separate between-taxon comparison to Audubon's Warbler. The strongest pattern of IBD is (**B**) within Audubon's Warbler and (**C**) between Audubon's and Myrtle warblers. Some IBD is observed between Audubon's and Black-fronted warblers, although some of this pattern is presumably driven by the most northern Audubon's populations likely experiencing some gene flow from Myrtle Warbler (Brelsford et al. 2011).



APPENDIX FIGURE 16. The spatial relationship between Audubon's and Black-fronted warblers. The plot shows PC2 from a PCA excluding Goldman's Warbler. Each point is shown relative to the latitude at which the bird was sampled, color-coded by taxon (red: Audubon's; yellow: Black-fronted).